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SOURCES OF CARBOHYDRATE FOR GERMINATION AND GROWTH OF ORCHID SEEDLINGS

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Ever since 1840, when Linck observed the endophytic fungus in orchid roots, the symbiotic relationship between these interesting plants has been studied by mycologists, plant physiologists, and horticulturists. We may roughly divide the research that has been done since 1840 into two main periods.

The first period consisted in efforts to verify the universality of the endophytic infection and to link this mycorrhizal condition with various phenomena of germination. Wahrlich ('86) was the first to establish the generality of the occurrence of the endophyte. He examined the roots of five hundred species of orchids and found all to be contaminated with a fungus which he held to be species of *Nectria*. Since the time of Wahrlich, the taxonomy of the fungal symbiont has passed through a complex and varied evolution, but a discussion of this phase of orchid research is beyond the scope of the present paper. The existence of the endophyte was further verified by Prillieux ('56, '60), Prillieux and Rivière ('56), and by Fabre ('56).

Bernard ('00) early suspected the relationship between the germination of orchid seeds and the presence of the endophyte. He obtained good germination on sawdust which was kept in the same greenhouse as the parent plants, and pointed out that this was probably due to an infection of the sawdust by the fungus from the parent plants. The publication of these results was followed by a number of other papers ('02, '03, '04, '06),

which further substantiated his opinions concerning the obligate relation between germination and the presence of a suitable fungus.

Burgeff ('09), in a monographic study of the various orchid endophytes, concluded, as had Bernard, that orchids were obligate symbiotic plants. He failed to recognize at that time the significance of his own experiment in which *Laelio-Cattleya* seeds germinated on a 0.33 per cent sucrose solution in the dark. The plants lived but ten months and further development either in the light or dark was impossible without the fungus. Had these cultures been exposed to light from the beginning, it is probable that he would have discovered the necessity of sugar rather than of the infected condition for normal development.

The second period of research began in a controversy over the possibility of germinating orchid seeds entirely asymbiotically. A complete review of this phase of the problem is without scientific interest. The writer cannot refrain, however, from quoting a sentence from Costantin ('26), which represents the resistance to the natural development of opinions from those of Wahrlich, Bernard, and Burgeff on the presence of the endophyte, to the latter views of Knudson on carbohydrate metabolism. Costantin said that Bultel was correct in saying that asymbiotic plants were normal externally, but—"Est-elle normale intérieurement? Non, si le champignon est absent: puisque sa présence est un des caractères de la vie normale de l'Orchidée."

The research dealing with the carbohydrate nutrition of plants grown entirely asymbiotically centers in the work of Knudson. Previous to Knudson, Bernard had obtained, in a few cases, germination of *Cattleya* and *Laelia* seeds asymbiotically on concentrated solutions of salep. He even suggested that some such method might be developed commercially. Salep is a preparation obtained by reducing the dried tubers of certain orchids to powder and contains (Knudson, '22) 48 per cent mucilage, 27 per cent starch, 5 per cent protein, and probably some sugar and mineral matter. However, it remained for Knudson ('22, '24, '25, '26, '27) to point out that the true significance of the mycorrhizal condition was in furnishing a source of carbohydrate to the orchid embryo and in maintaining a favor-

able degree of acidity. A paper by Knudson ('16), previous to his work on orchids, called attention to the fact that sugars had a favorable influence on the growth of higher plants. The results obtained in this work, together with data taken from Bernard and Burgeff, suggested that soluble organic substance might cause germination. Germination was obtained on an agar culture prepared by autoclaving 400 gms. of dormant canna tubers with 600 cc. of water. Germination also occurred, with varying degrees of excellence, on peat agar, carrot-root extract, beet extract, and sugars. A complete mineral nutrient solution to which sugars were added gave good development. Fructose and glucose were the sugars used, fructose proving to be the better.

Germination and growth were obtained asymbiotically with the addition of sugars by Clement ('24 a, b, '26, '29, '32), Ballion and Ballion ('24, '28), and Bultel ('24–'25, '26). Bultel ('25) mentioned that fructose was preferable to glucose, but no experimental data were given.

While the above authors, building chiefly on the work of Knudson, sufficiently established the possibility of growing normal plants in the presence of sugar in the absence of the endophyte, La Garde ('29) was the first to study systematically the comparative value of the different sugars. Using 2 per cent solutions of maltose, glucose, fructose, and sucrose, he found their comparative value to be maltose > fructose > glucose > sucrose. A year later, Quednow ('30) published his observations on a more extended list of sugars. He found the order of excellence to be glucose > fructose > sucrose > maltose > mannite > galactose > lactose. Smith ('32) used sucrose, glucose, and maltose, singly and in all sorts of combinations, and observed no apparent difference in the growth of the seedlings.

Unusual facilities for research have enabled the writer to extend the list of sugars which has been used for orchid germination to include the rarer and more expensive forms. This opportunity, together with the discrepancies between the work of La Garde, Quednow, and Smith, led to the present work. The sugars were added to three different complete mineral nutrient solutions in amounts to give 7 gms. of carbon per liter. The compositions of these solutions were as follows:

Knudson's ('22) Solution		Shive's ('15) Solution		La Garde's ('29) Solution	
$MgSO_4.7H_2O$	.250 gm.	$MgSO_4.7H_2O$	4.930 gm.	$MgSO_4.7H_2O$	1.00 gm.
$Ca(NO_3)_2.4H_2O$	1.000 gm.	$Ca(NO_3)_2.4H_2O$	1.228 gm.	$Ca(NO_3)_2.4H_2O$	1.00 gm.
$(NH_4)_2SO_4$	.500 gm.	KH_2PO_4	1.960 gm.	KH_2PO_4	1.00 gm.
K_2HPO_4	.250 gm.			$CaCl_2$	1.00 gm.
				NH_4NO_3	.50 gm.
				$(NH_4)_2CO_3.H_2O$	.500 gm.

Iron was added in all cases as 10 cc. of a M/200 suspension of $FePO_4$ prepared according to Livingston ('19) in a liter of nutrient solution. Both Knudson and La Garde added iron in such quantities as to cause a heavy precipitate of iron phosphate. La Garde states that this precipitate was filtered off before the final sterilization and contained, besides iron and phosphate ions, calcium and potassium. It seems unwise to cause this bulky precipitate because it tends to adsorb other ions which are removed with it in filtration. The quantity of $FePO_4$ added by the author does not cause appreciable precipitation at the hydrogen-ion concentration used. This reduced amount is undoubtedly sufficient in quantity, since it is ten times that originally recommended by Livingston. La Garde designates the iron compound used by him as $Fe_3(PO_4)_2.8H_2O$. Knudson ('22), Quednow ('30), and Smith ('32) also added iron as ferrous phosphate. Since it is well known that the ferrous ion in the presence of oxygen reduces nitrate to nitrite, the ferric ion was used in the present work.

The solutions were made up in liter flasks, and adjusted to pH 4.00 with HCl. All precipitate dissolved at this acidity, but there was a slight opalescence due to ferric phosphate. The solutions were then titrated by means of the quinhydrone electrode to such pH (see tables) that the values after sterilization were 4.8–5.1. Aliquots of 100-cc. portions were placed in 200-cc. Erlenmeyer flasks and 1.75 grams of Merck's Reagent Powdered Agar added. Sterilization was by autoclaving at twenty pounds pressure for twenty minutes. The medium was allowed to solidify in a slanting position.

It is extraordinarily difficult to maintain sterile cultures in warm moist atmospheres over long periods of time, and after many preliminary failures, the following plan was adopted. The solutions were added to the culture flasks through a funnel, care

being taken not to moisten the necks of the flasks. The flasks were then closed with cotton plugs. A duplicate set of cotton stoppers was carefully and tightly rolled, sterilized in empty flasks in the autoclave, and then immediately transferred to the dry-air oven until thoroughly dry. The seeds were shaken vigorously for thirty minutes in a small vial of calcium hypochlorite prepared as recommended by Wilson ('15). This vial was then clamped in a sloping position so that contaminating substances might not fall in from the air. A culture flask and an empty flask containing the especially prepared cotton plug were held in a horizontal position in the left hand. The temporary cotton plug was withdrawn from the culture flask and dropped. A platinum-loop inoculating needle, held in the right hand, was quickly flamed and a loopful of seeds transferred directly from the hypochlorite solution to the drop of moisture that always exudes from the solidified agar. The neck of the flask was then flamed and the especially prepared stopper quickly drawn from the blank flask and inserted in the culture flask. This procedure is advisable as it involves a minimum of exposure of the cotton plug that is finally used in the culture flask, and insures its perfect dryness.

The drop of moisture containing the seeds on the edge of the agar was then distributed around the entire margin by rotating the flask carefully. This even distribution of seeds was maintained by placing the flask in a rack so constructed that the agar surface was perfectly level, thus preventing the liquid drop from draining to one side and carrying the seeds with it. After a convenient number of flasks had been inoculated, the necks of the flasks were again flamed and the cotton plug well charred on the surface. The plugs and the outer surface of the necks were then moistened with saturated $HgCl_2$ solution. Heavy waxed paper was then dipped in the $HgCl_2$ solution, tightly wrapped around the stoppers and the upper part of the flask, and held firmly in position by rubber bands. The writer has found that unless these precautions are taken, fungi will frequently grow along the surface of the flask and penetrate the stopper, contamination usually not appearing until three or four months after inoculation. Bernard, certainly a well-trained and experienced

mycologist, has commented on the extraordinary difficulty of maintaining orchid cultures sterile in the moist warm atmosphere desirable for germination. By taking the above precautions, the writer has maintained sterile cultures as long as three years in moisture-saturated atmosphere at 25–35° C.

In every case, the cultures were prepared in triplicate. The seeds for the entire series were from a single pod of *Cattleya Trianae* Linden & Rchb. f. The flower was pollinated November

TABLE I

DATA OF KNUDSON'S SOLUTION

Sugar	pH adjusted before sterilization	pH at time of planting	pH after supporting growth 8 months
d-glucose	4.12	5.1	4.2
d-fructose	4.12	4.9	3.8
d-galactose	4.12	5.0	No growth
d-mannose	4.13	5.0	4.2
l-xylose	4.15	5.0	No growth
l-arabinose	4.10	5.0	No growth
Maltose	4.15	5.1	4.4
l-rhamnose	4.15	5.0	No growth
Sucrose	4.15	5.1	4.4
Raffinose	4.12	4.8	4.4

TABLE II

DATA OF SHIVE'S SOLUTION

Sugar	pH adjusted before sterilization	pH at time of planting	pH after supporting growth 8 months
d-glucose	4.12	5.1	4.4
d-fructose	4.12	4.9	4.0
d-galactose	4.12	5.0	No growth
d-mannose	4.13	5.0	4.5
l-xylose	4.15	5.0	No growth
l-arabinose	4.10	5.0	No growth
Maltose	4.15	5.1	4.6
l-rhamnose	4.15	5.0	No growth
Sucrose	4.15	5.1	4.5
Raffinose	4.12	4.8	4.4

TABLE III

DATA OF LA GARDE'S SOLUTION

Sugar	pH adjusted before sterilization	pH at time of planting	pH after supporting growth 8 months
d-glucose	4.25	4.9	3.5
d-fructose	4.24	4.8	3.8
d-galactose	4.22	4.8	No growth
d-mannose	4.24	4.9	3.8
l-xylose	4.24	4.9	No growth
l-arabinose	4.24	4.9	No growth
Maltose	4.25	4.9	4.2
l-rhamnose	4.22	4.9	No growth
Sucrose	4.24	4.9	4.1
Raffinose	4.51	4.9	4.2

27, 1930, and the matured pod harvested March 11, 1932, after a developmental period of over sixteen months. The inoculation of the culture media was made June 15, 1932, and the observations were recorded February 25, 1933, after a growth period of about eight months.

In the younger stages of development the diameter of the protocorm is an accurate basis for comparative determinations of growth, but after the seedling has developed leaves the growth is largely vertical rather than mere enlargement of the nearly round protocorm. Accurate measurements of the height of the young plant are difficult to obtain, since it is too large to measure by a microscope micrometer and too small for any less accurate means. For these reasons, seedlings as old as eight months can best be rated with the eye by comparing different culture flasks and sorting them into a few groups. The results of such a comparison after the growth period of eight months are shown in table IV. The relative excellence of the cultures is designated by the number of X's.

The results show that d-mannose produced definitely the best growth. This is followed by the group glucose-maltose-fructose, and this in turn by the third group sucrose-raffinose, and then l-xylose. No growth was obtained on d-galactose, arabinose, or rhamnose. A comparison of these results with the molecular

TABLE IV

GROWTH DATA

Description of plants	Knudson's Mineral Solution		Shive's Mineral Solution		La Garde's Mineral Solution	
	Sugar	Growth	Sugar	Growth	Sugar	Growth
Group I Exceptionally good growth and chlorophyll development	d-mannose	xxxx*	d-mannose	xxxxx	d-mannose	xxxxx
Group II Moderately good growth and chlorophyll development	d-glucose	xxx	d-glucose	xxx		
	maltose	xxx	maltose	xxx	maltose	xxx
	d-fructose	xxx	d-fructose	xxx	d-fructose	xxx
			sucrose	xxx		
Group III Poor growth and chlorophyll development	sucrose	xx			sucrose	xx
	raffinose	xx	raffinose	xx	raffinose	xx
Group IV No growth	l-xylose	x			l-xylose	x
	d-galactose	0	d-galactose	0	d-galactose	0
	l-arabinose	0	l-arabinose	0	l-arabinose	0
	l-rhamnose	0	l-rhamnose	0	l-rhamnose	0

*The number of x's denotes the relative excellence of the cultures.

configuration of the respective sugars indicates that in but one physical characteristic do they show any consistent physiological action—and that is that the pentoses do not allow the germination and growth of orchid seedlings. It is not impossible that their quality of being levo-rotatory is related to their physiological reaction. The two instances in which l-xylose allowed growth are of doubtful authenticity. Only two or three seeds germinated in each flask, and these never developed beyond a very rudimentary protocorm. Such rudimentary development was occasionally noted even on sugar-free agar cultures, but in those cases also development never proceeded beyond a rudimentary protocorm. It is interesting to note that rhamnose, although it

has six carbon atoms, is structurally a methylated pentose and reacts physiologically to orchid seedlings as a pentose. Galactose, although an aldo-hexose, as is also d-mannose, supported no growth. These results are in approximate agreement with those of Quednow, cited above.

From the following considerations the author believes that the conspicuous superiority of d-mannose is of especial significance. Mannose, in the form of mannan, is known to be widely distributed as a constituent of the cell wall of many plants. This is particularly true of seeds (Onslow, '23). For example, various complex mannans have been found in the seeds of palms, asparagus, clover, coffee bean, onion, and various Leguminosae, Coniferae, and Umbelliferae. Mucilages are particularly rich in mannans, as, for instance, those obtained from lily bulbs (Parkin, '01) and tubers of various genera of Orchidaceae. Pringsheim and his coworkers ('24, '28) succeeded in isolating and studying mannan from orchid tubers. In this instance, it was water soluble and was precipitated by alcohol as a white powder. Klein ('32) gives a procedure for isolating mannan from salep itself.

Salep, a product of orchid tubers, has been used as a substrate for the orchid fungi and for germinating orchid seeds in the presence of some symbiotic fungus from the earliest days of orchid research. It therefore appears fairly certain that the fungal element of the orchid mycorrhiza is able to hydrolyze mannan to soluble mannose. In this way the symbiotic fungi could make available any mannose which might be present in the woody and mossy substrate of epiphytic orchids in their natural habitat. The extraordinary slowness of the development of orchid seedlings would be supported by the presence of only very small quantities of mannan.

The Missouri Botanical Garden has produced seedlings by the symbiotic method that surpassed in quality anything that the author has seen obtained by asymbiotic methods. In many instances the agar substrate consisted of the usual mineral substances and finely shredded cocoanut fiber. An appropriate fungus was inoculated on this medium some time before the seeds were sown. Seedlings grown on fungus-inoculated cocoanut-

fiber substrate always surpassed those grown on La Garde's maltose media. Bultel ('25) also found that symbiotic cultures gave superior results with all genera tested by him except Phalaenopsis. The nature of his substrate was not indicated.

The writer has already reported ('33) on the superior value of the La Garde mineral solution over other solutions, and it seems scarcely probable that symbiotic cultures could owe their superiority to any inorganic constituent. It is much more probable that the carbohydrate relationship is the important feature. Knudson has shown the effectiveness of orchid fungi in hydrolyzing starch to available sugar. Might not the superiority of the symbiotic cultures be due to the mannose produced by hydrolysis from the cocoanut fiber?

In order to determine the actual presence of mannans in cocoanut fiber, an analysis was carried out according to the method of Haegglund and Klingstedt ('24, '27). Ten gms. of cocoanut fiber were allowed to stand in 150 cc. of 72 per cent H_2SO_4 for $2\frac{1}{2}$ days. The mixture was then diluted with water and the acid neutralized with $CaCO_3$. The precipitate was filtered off, the residue washed on a suction filter, and the combined filtrate and washings were evaporated on the steam bath to 150 cc. Then H_2SO_4 was added to give a 2 per cent solution and the mixture boiled 2 hours. The acid was again neutralized with $CaCO_3$, then weakly acidified with $HC_2H_3O_2$, and evaporated to 100 cc. After cooling, 10 cc. of phenylhydrazine plus 20 cc. water were added and allowed to stand several days. A very definite precipitate of the insoluble phenylhydrazone was obtained, indicating the presence of mannose.

The above method is scarcely quantitative because of the difficulty of obtaining complete hydrolysis of mannans without causing their oxidation at the same time. The very heavy precipitate of $CaSO_4$ which is produced when the acid is neutralized with $CaCO_3$ is bulky and difficult to wash thoroughly.

The seedlings grown on La Garde's maltose solution equalled those of the same age originally obtained by La Garde. Hence the mannose cultures might be regarded as distinctly superior to those yet obtained by the use of a purely synthetic medium and approach in quality the best of the symbiotic cultures. It

is to be regretted, however, that this could not be verified positively by comparison, since seedlings of the same age grown symbiotically were not at hand.

The glucose cultures were also of high quality, particularly as to their green color. No trace of yellowness or of inferior chlorophyll development was observed in seedlings grown on this sugar.

It must be noted that superiority of symbiotic cultures may depend not only on a carbohydrate relation, but also on a favorable degree of acidity. Knudson has pointed out that satisfactory fungal symbionts maintain a favorable pH of the media for the germination of the seeds. The pH of asymbiotic media can, of course, be adjusted artificially, but the initial favorable acidity is difficult to maintain over long periods of time.

The author hopes that some worker equipped to carry both symbiotic and asymbiotic cultures simultaneously will test further the suggestion that symbiotic cultures owe their superiority to mannose and to the constantly favorable pH relationship.

SUMMARY

1. The growth of orchid seedlings over a period of eight months was observed on a series of sugars, each added to three different inorganic media in amounts to give seven grams of carbon per liter. The order of excellence of growth on the different sugars was: d-mannose > d-glucose > maltose > d-fructose > sucrose > raffinose.

2. No growth was obtained on d-galactose, and the pentoses, l-arabinose, l-rhamnose, and l-xylose. This inability of the pentose sugars to support growth may be related to their levorotating property. Galactose occupies an anomalous position.

3. Mannose gave conspicuously the better growth. This may be related to the fact that symbiotic cultures containing cocoanut fiber, an effective source of mannan, produce seedlings superior to any that the author has seen produced asymbiotically.

4. The mineral nutrient medium of La Garde plus d-mannose is regarded as the best asymbiotic culture medium for orchids, as shown by the reaction of *Cattleya Trianae* Linden and Rchb. f. seedlings.

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